AUTHORITY: 21 U.S.C. 357.

SOURCE: 39 FR 18944, May 30, 1974, unless otherwise noted.

Subpart A—Definitions; Interpretations; Requirements

§ 436.1 Sterility requirements of items packaged with sterile antibiotic drugs.

(a) *Diluents packaged in combination with sterile antibiotic drugs.* If a sterile antibiotic drug is packaged in combination with an immediate container of a diluent, the immediate container of diluent shall be sterile when tested by the method prescribed in § 436.20(e)(1).

(b) *Dispensers packaged in combination with sterile antibiotic drugs.* If a sterile antibiotic drug is packaged in combination with a dispenser, such dispenser shall be sterile when tested by the method prescribed in § 436.20(e)(1).

[39 FR 18944, May 30, 1974, as amended at 41 FR 46852, Oct. 26, 1976]

§ 436.2 Alternative assay methods.

Alternative assay methods (including automated procedures) employing the same basic chemistry or microbiology as the official methods described in this part and in the individual monographs of this chapter may be used, provided the results obtained are of equivalent accuracy. However, only the results obtained from the official methods designated in the individual monographs are conclusive.

Subpart B—Sterility Test Methods

§ 436.20 Sterility test methods and procedures.

(a) *Laboratory facilities.* The test must be performed using aseptic techniques in an area as free from contamination as is possible to achieve. Testing should not be conducted under direct exposure to ultraviolet light or in areas under aerosol treatment. Environmental tests to assess the suitability of testing conditions should be made frequently enough to assure the validity of test results.

(b) *Equipment and reagents*—(1) *Bacterial membrane filter.* The filter has a nominal porosity of 0.45 micron±0.02 micron, a diameter of approximately 47 millimeters, and a flowrate of 55 milliliters to 75 milliliters of distilled water passing each square centimeter of filter area per minute with a differential pressure of 70 centimeters of mercury at 25° C.

(2) *Penicillinase solutions.* When the amount of penicillinase to be used is specified in terms of Levy units, use a penicillinase solution standardized in terms of Levy units. One Levy unit of penicillinase inactivates 59.3 units of penicillin G in 1 hour at 25° C. and at a pH of 7.0 in a phosphate buffered solution of a pure alkali salt of penicillin G when the substrate is in sufficient concentration to maintain a zero order reaction.

(c) *Culture media.* Use ingredients that conform to the standards prescribed by the U.S.P. or N.F. In lieu of preparing the media from the individual ingredients, they may be made from dehydrated mixtures which, when reconstituted with distilled water, have the same or equivalent composition as such media and have growth-promoting buffering, and oxygen tension-controlling properties equal to or better than such media. The pH of each medium should be adjusted with 2*N* hydrochloric acid or sodium hydroxide

before sterilization, so that after sterilization and the addition of the penicillinase, if necessary, the pH will fall within the specified range. Dispense 90±10 milliliter quantities of the liquid media into individual test tubes (38 millimeters x 200 millimeters). Close the tubes with suitable closures, and sterilize in an autoclave at 121° C. for 20 minutes. The autoclave temperature should be reached within 10 minutes. After sterilization, cool the medium at once to approximately 25° C. and store at 20° C. to 30° C. The sterility of each lot of tubes of liquid medium may be confirmed by incubating an adequate number of tubes as described in the test procedures in paragraph (e) of this section.

(1) *Medium A.* Use U.S.P. fluid thioglycolate medium I.

(2) *Medium B.* Use U.S.P. fluid thioglycolate medium I, with sufficient sterile penicillinase added to inactivate the penicillin activity in the sample under test. The penicillinase must be added to individual tubes of sterile medium A, using aseptic technique. Prior to use, or at the time of the test, a representative number of the tubes containing added penicillinase are incubated at 30° –32° C. for 24 hours to 48 hours, and are examined for sterility. If the sample contains penicillin as the only antibiotic, the ability of the penicillinase to inactivate all the penicillin in the sample under test is checked as follows: Add to one test tube of medium B the proper amount of penicillin from one of the individual containers under test. Then add 1.0 milliliter of a 1:1.000 dilution of an 18–24 hour culture of *Staphylococcus aureus* (American Type Culture Collection 6538–P)[1] in medium A. Typical microbial growth must be observable after 24 hours incubation at 30°–32° C. If the sample contains a mixture of penicillin plus some other antibiotic or antibacterial agent the ability of the penicillinase to inactivate all the penicillin in the sample is not tested directly on the sample under test, but is determined separately, using an amount of penicillin alone equivalent to the amount of penicillin in the sample or by any other suitable method for standardizing the penicillin-inactivating power of the penicillinase preparation.

(3) *Medium C.* To each liter of medium A add 5.0 milliliters of polysorbate 80 before sterilization. To each tube of sterilized medium add sufficient sterile penicillinase, and proceed as directed for medium B.

(4) *Medium D.* To each liter of medium A add 5.0 milliliters of polysorbate 80 and sufficient 2*N* sodium hydroxide so that the pH will be 7.9±0.1 after sterilization. Then add sufficient sterile penicillinase to each tube and proceed as directed for medium B.

(5) *Medium E.* Use U.S.P. XVIII soybean-casein digest medium.

(6) *Medium F.* To each liter of medium E add 5.0 milliliters of polysorbate 80 before sterilization. To each tube of sterilized medium add sufficient sterile penicillinase to solubilize the penicillin in the sample to be tested.

(7) *Medium G.* Prepare as follows:

Peptic digest of animal tissue .....6.0 gm.
Pancreatic digest of casein .....4.0 gm.
Yeast extract .....3.0 gm.
Beef extract .....1.5 gm.
Dextrose.....1.0 gm.
Agar.....15.0 gm.
Distilled water, q.s.....1,000.0 ml.
pH 6.6±0.1

Suspend the powder in a liter of distilled water. Allow to stand for 5 minutes, then mix thoroughly. Boil for 1 or 2 minutes or until solution is complete. Dispense in suitable flasks and sterilize at 121° C. for 15 minutes. Aseptically pour approximately 25-milliliter quantities into sterile Petri dish bottoms measuring 20 millimeters x 100 millimeters. Cover plates with sterile porcelain tops, glazed on the outside. Allow plates to stand at room temperature for 48 hours prior to use as a control on the sterility of the plates.

(8) *Medium H.* Prepare, sterilize, and dispense as described for medium G, except as follows:

Dextrose .....40.0 gm.
Peptic digest of animal tissue .....10.0 gm.
Agar.....15.0 gm.
Distilled water q.s.....1,000.0 ml.
pH 5.6±0.1 after sterilization

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

(9) *Medium I.* To each liter of Medium A add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol.

(10) *Medium J.* To each liter of Medium E add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol.

(11) *Medium K.* (*Rinse medium*). Prepare as follows:

Peptic digest of animal tissue	5.0 gm.
Beef extract	3.0 gm.
p-tert-octylphenoxy polyethoxyethanol	10.0 gm.
Distilled water, q.s	1,000.0 ml.

pH 6.9±0.2 after sterilization

(12) *Medium L.* To each liter of Medium A add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol and approximately 10,000 Levy units of penicillinase.

(13) *Medium M.* To each liter of Medium E add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol and approximately 10,000 Levy units of penicillinase.

(14) *Medium N:*

Pancreatic digest of casein	15.0 gm.
Peptic digest of soybean meal	5.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Water	1,000.0 ml.

pH 7.3±0.2 after sterilization

(d) *Diluting fluids*—(1) *Diluting fluid A.* Dissolve 1 gram of U.S.P. peptic digest of animal tissue or equivalent in sufficient distilled water to make 1,000 milliliters. Dispense in flasks and sterilize as described in paragraph (c) of this section. Final pH=7.1±0.1.

(2) *Diluting fluid B.* To each liter of diluting fluid A add 5.0 milliliters of polysorbate 80 before sterilization.

(3) *Diluting fluid C.* To each liter of diluting fluid A add 0.5 gram of sodium thioglycollate, and adjust with NaOH so that after sterilization the final pH will be pH 6.6±0.6. Dispense in flasks and sterilize as described in paragraph (c) of this section.

(4) *Diluting fluid D.* To each liter of diluting fluid A add 1 milliliter of *p-tert*- octylphenoxy polyethoxyethanol. Dispense in flasks and sterilize as described in paragraph (c) of this section. Final pH=7.1±10.1.

(5) *Diluting fluid E.* Use isopropyl myristate that is sterile and that has a water-extract pH of 5.5 or greater. Determine the water-extract pH of a portion of the isopropyl myristate as follows: Place 100 milliliters of the isopropyl myristate sample and 10 milliliters of distilled water into a centrifuge bottle of approximately 250 milliliters capacity and seal the bottle tightly. Place the centrifuge bottle on a shaker so that its longest dimension is oriented in the direction of shaker movement and shake at 250 cycles per minute for 1 hour. Centrifuge the bottle at 1,800 revolutions per minute for 20 minutes. With a suitable vacuum system, remove and discard the upper layer; then pipet 5 milliliters of the lower water layer into a beaker and determine the pH using a standardized pH meter. If the water-extract pH is less than 5.5, pass the isopropyl myristate through a glass column packed with basic aluminum oxide, activity grade No. 1. Determine the water-extract pH of a portion of the isopropyl myristate that has been passed through the aluminum oxide column. Sterilize isopropyl myristate by filtration through a 0.22-micron membrane filter and aseptically dispense 100-milliliter portions into sterile 250-milliliter flasks.

(6) *Diluting fluid F.* To each liter of diluting fluid A add 20 grams of disodium edetate, and adjust with NaOH so that after sterilization the final pH will be 7.1±0.1. Dispense in flasks and sterilize as described in paragraph (c) of this section.

(7) *Diluting fluid G.* To each liter of sterile diluting fluid A add 10 grams of sterile *L*-lysine.

(8) *Diluting fluid H.* To each liter of diluting fluid A add 10 grams of sodium bicarbonate before sterilization.

(9) *Diluting fluid I.* To each liter of diluting fluid A add 23.4 grams of sterile L-arginine base.

(10) *Diluting fluid J.* Sterilize 2.0 grams of anhydrous sodium carbonate by dry-heating at 180° C for 2 hours. Dissolve in 100 milliliters of diluting fluid A just prior to use.

(e) *Conduct of test*—(1) *Bacterial membrane filter method*—(i) *Sample preparation*—(*a*) Antibiotic drug. From each of 20 immediate containers, aseptically transfer approximately 300 milligrams of solids if it is not a liquid drug, or 1 milliliter by volume if it is a liquid drug, or the entire contents if the container contains less than these amounts; except that if it is a liquid

drug containing penicillin in a concentration greater than 300,000 units per milliliter, use the volume that contains 300,000 units, into a sterile 500-milliliter Erlenmeyer flask containing approximately 200 milliliters of diluting fluid A. (If it is a composite sample packaged in one immediate container in accordance with the requirements of § 431.5(b) of this chapter, transfer the entire contents, or approximately 6 grams, into the Erlenmeyer flask.) Stopper the flask and swirl to dissolve the drug. As soon as the sample has completely dissolved, proceed as directed in paragraph (e)(1)(ii) of this section. If the pooled portions from 20 containers will not dissolve completely in 200 milliliters of diluting fluid or will not filter rapidly, 400 milliliters of diluting fluid may be used or two separate tests may be performed using a pool of 10 containers for each test.

(*b*) Diluent packaged in combination with a sterile drug. Using the entire contents from each of 20 immediate containers, proceed as directed in paragraph (e)(1)(ii) of this section.

(*c*) Sterile dispensers packaged in combination with a sterile drug. Prepare 20 clean, empty containers of approximately the same size as those in which the sterile antibiotic drug is packaged. To each container add diluting fluid A in a volume approximately the same as that of the sterile drug when it is prepared for dispensing. Cap the containers, sterilize by autoclaving at 121° C. for 20 minutes, and then allow to cool to room temperature. Aseptically open each dispenser package and remove each dispenser in turn. Use each aseptically to remove 1 milliliter of the fluid from a separate sterile container prepared as described above. Aseptically transfer the fluid to a 500-milliliter Erlenmeyer flask containing approximately 200 milliliters of diluting fluid A. Stopper the flask and proceed as directed in paragraph (e)(1)(ii) of this section.

(ii) *Test procedure.* Aseptically filter the solution through a bacteriological membrane filter. All air entering the filtering system is filtered through air filters capable of removing microorganisms. Filter three 100-milliliter quantities of diluting fluid A through the membrane. For the penicillin and cephalosporin classes of antibiotics, add sufficient penicillinase to diluting fluid A to inactivate the residual antibiotic activity on the membrane after filtration. By means of a sterile circular blade, paper punch, or any other suitable sterile device, cut a circular portion (approximately 17.5 millimeters in diameter) from the center of the filtering area. Transfer the cut center area to a sterile 38 by 200 millimeter (outside dimensions) test tube containing 90±10 milliliters of sterile medium A. Incubate the tube for 7 days at 30°–32° C. Using sterile forceps, transfer the remaining outer portion of the membrane into a second similar tube containing 90±10 milliliters of medium E. Incubate the second tube for 7 days at 22°–25° C.

(2) *Direct method.* From each of 20 immediate containers, transfer approximately 300 milligrams of solids if it is not a liquid drug, or 1 milliliter by volume if it is a liquid drug, or the entire contents if it contains less than these amounts, except if it is a liquid drug containing penicillin in a concentration greater than 300,000 units per milliliter use that volume that contains 300,000 units, into individual sterile test tubes (38 millimeters × 200 millimeters) containing 90±10 milliliters of medium A. Incubate all tubes at 30° C. to 32° C. for 7 days. Gently agitate the tubes every 1 to 3 days or until complete solubilization occurs. At intervals, examine all tubes for visible growth. If growth is observed in any tube, confirm by microscopic examination. From each of the same 20 immediate containers, transfer a second portion (equivalent to that portion initially transferred to the tubes containing medium A) to individual sterile test tubes (38 millimeters × 200 millimeters) containing 90±10 milliliters of medium E, except when each container does not have sufficient material to provide for the two similar-size portions, obtain the second portion from 20 additional immediate containers. Incubate all tubes at 22° C. to 25° C. for 7 days. Gently agitate the tubes every 1 to 3 days or until complete solubilization occurs. At intervals, examine all tubes for visible growth. If growth is observed in any tube, confirm by microscopic examination.

(3) *Bacterial membrane filter method for ophthalmic ointments*—(i) *Ointments that do not contain penicillin.* From each of 10 immediate containers aseptically transfer 0.1 gram of the product into a sterile 250-milliliter flask containing 100 milliliters of diluting fluid E which has previously been heated to a temperature of 47° C. Repeat the process, using 10 additional containers. Swirl both of the flasks to dissolve the ointment. Immediately aseptically filter each solution through a separate bacteriological membrane filter previously moistened with approximately 0.2 milliliter of medium K. Filter all air entering the system through air filters capable of removing microorganisms. Remove any residual antibiotic from the membranes by rinsing each filter five times with 100 milliliters of medium K. The membranes should be covered with fluid throughout each step of the filtration procedure until the end of the last filtering step. By means of a sterile circular blade, paper punch, or other suitable sterile device, cut a circular portion (approximately 17.5 millimeters in diameter) from the center of the filtering area of each membrane. Transfer the center portion of the filtering area of each filter to a sterile test tube 38 millimeters × 200 millimeters (outside dimensions) containing 90 milliliters±10 milliliters of sterile medium I. Incubate the tube for 7 days at 30° C. to 32° C. Using sterile forceps transfer the outer portion of each filter to a similar test tube containing 90 milliliters ±10 milliliters of sterile medium J. Incubate this tube for 7 days at 22° C. to 25° C.

(ii) *Ointments containing penicillin.* Proceed as directed in paragraph (e)(3)(i) of this section, except in lieu of sterile medium I use sterile medium L for the center portion of the filtering area of each filter and in lieu of sterile medium J use sterile medium M for the remaining outer portion of each filter.

(f) *Evaluation of results*—(1) *Bacterial membrane-filter method.* The batch, or the part of the batch represented by a particular filling operation meets the requirements of the test if no sample tube shows growth. If growth is observed in any sample tube, run a second test in the appropriate medium, except perform it in duplicate, using 40 immediate containers. If in the original test, growth is observed in only one of the two media, test both portions of the cut filter membrane by placing each into a separate tube of the same medium. The batch meets the requirements if no tube on the second test shows growth. If growth is observed in any of the control tubes as well as in the sample tubes in either the original or the second test such test is invalid and must be performed again. In any event, further tests may be justified if there is sufficient reason to believe that the results obtained in the first and second tests may not be valid. In such instances, the batch is satisfactory if on the final test no tube shows growth.

(2) *Direct method.* The batch, or the part of the batch represented by a particular filling operation, meets the requirements of the test if no tube shows growth after incubation. If growth is observed in any sample tube, run a second test in the appropriate medium using 40 immediate containers. The batch is satisfactory if, on the second test, no tube shows growth. If growth is observed in any of the control tubes (except inoculated tubes, if the sample is penicillin) as well as in the sample tubes in either the original or the second test, such test is invalid and must be performed again. In any event, further tests may be justified if there is sufficient reason to believe that the results obtained on the first and second tests may not be valid. In such instances the batch is satisfactory if in the final test no tube shows growth.

[39 FR 18944, May 30, 1974, as amended at 41 FR 46852, Oct. 26, 1976; 42 FR 27228, May 27, 1977; 43 FR 43457, Sept. 26, 1978; 49 FR 25846, June 25, 1984; 49 FR 40006, Oct. 12, 1984; 50 FR 48397, Nov. 25, 1985; 52 FR 4611, Feb. 13, 1987; 53 FR 13401, Apr. 25, 1988]

Subpart C—Biological Test Methods

§ 436.31 Equipment and diluents for use in biological testing.

(a) *Equipment*—(1) *Temperature-measuring devices.* Use an accurate clinical